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THE ANTIBACTERIAL POTENTIAL OF FERMENTED TARO AND ITS DEVELOPMENT AS A FOOD PRESERVATIVE

by
Wayne S. Muller
Alfred L. Allen
Anthony Sikes
and
Andy Senecal

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U.S. Army Soldier and Biological Chemical Command
Soldier Systems Center
Natick, Massachusetts 01760-5020

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14. ABSTRACT

In an attempt to revitalize the Hawaiian economy, congressional funds have been allocated to develop industries and products associated with Hawaii. One such product is poi, a purplish to grayish paste made of ground taro, a traditional staple food in Hawaii. Taro/poi exhibits many unique properties to be explored in this program. This interim report covers one aspect of this research: The antibacterial potential of the fermented taro and its development as a food preservative. In an attempt to understand the natural fermentation of taro to poi, bacteria have been isolated from freeze dried poi produced in Hawaii. Bacteria believed to be involved in the fermentation have been isolated and identified. It was determined that taro can support the growth of bacteriocin-producing bacteria. Bacteriocins are small peptides that are naturally produced by food-safe organisms that can inhibit food spoilage/pathogenic bacteria. A relatively dilute solution of taro is needed to support the growth of the bacteria and the production of the bacteriocin. One bacteriocin has been produced in relatively large quantities with a high activity level. The bacteriocin's activity in taro was stable. The fermented taro containing the bacteriocin will be evaluated in rations to determine its effectiveness as a food preservative against various food spoilage/pathogenic bacteria.

15. SUBJECT TERMS

POI	FOOD PRESERVATION	SPOILAGE	FOOD PRESERVATIVE
TARO	BACTERIOCINS	FOOD SPOILAGE	PATHOGENIC MICROORGANISMS
HAWAII	PATHOGENIC BACTERIA	FUNCTIONAL FOOD	

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Preface

This is a report on one part of an integrated project performed under the Biosystems Technology Program (BTP), program element number PE 63001, performed from August 2000 to September 2001. The BTP is congressional funding that focuses on environmentally preferable and responsible products and services derived from tropical plants and microorganisms. The funded project is entitled "Development of Taro/Poi into Military/Commercial Functional Foods." This report covers one part of the project: Phase I of the functionality studies, the antibacterial potential of the fermented poi process/product for bacteriocin production. The project involved several team members, including two microbiologists, a food scientist and a chemical engineer at the lead federal agency, Natick Soldier Systems Center, Natick, MA. The goal is to determine if fermented taro can be developed into an effective food preservative.

Based on this research a U.S. Patent has been filed on March 19, 2002 titled "Method For Making A Food Preservative And For Preserving Food".

SUMMARY

Congressional funds have been appropriated to revitalize the economy in Hawaii by developing products unique to that state. Taro, a native crop of Hawaii, has many interesting properties as well its fermented product poi. The program funded evaluates the functional properties of taro/poi, its application into existing products and its development into unique military/commercial food items. This interim report covers one aspect of the program: the antibacterial potential of the fermented taro and its development as a potential food preservative.

In sampling freeze-dried poi from Hawaii, researchers isolated a number of bacteria. Preliminary identification has been made of bacteria that may be responsible for the fermentation of the taro to sour poi. As a medium, taro has been shown to support good growth of bacteria that produce bacteriocins. The concentration of taro necessary to support growth and bacteriocin production is relatively low (1 % taro). It was determined that taro may not support bacteriocin production from all bacteria without supplementing the taro. A 5 % taro solution supported good growth of *Streptococcus sp ATCC 10034* but the bacteriocin, diplococcin, was not produced. However, by supplementing the taro medium with a nitrogen source, such as trypticase or yeast extract, the bacterium was able to produce the bacteriocin. The bacteriocin nisin was produced in 5 % taro and the activity level was determined to be 8,000 – 10,000 AU/ g against the test organism *Micrococcus luteus*. Three liters of the 5 % liquid culture was freeze- dried, producing approximately 100g of taro containing nisin. Before and after freeze drying the taro the nisin activity level remained the same indicating stability of the bacteriocin. This material will be tested in rations to determine if it is effective against food spoilage/pathogenic bacteria.

The plan for the upcoming year is to continue to isolate and identify bacteria in the natural fermented taro and determine if any of the isolates exhibit antibacterial properties. Nisin, as well as other bacteriocins, will be produced in the taro. These fermented products will be incorporated into military ration components to evaluate their efficacy as food preservatives.

THE ANTIBACTERIAL POTENTIAL OF FERMENTED TARO AND ITS DEVELOPMENT AS A FOOD PRESERVATIVE

INTRODUCTION

Taro and its natural fermented end product, poi, are natural products native to tropical and sub-tropical regions such as Hawaii. These products have displayed very little commercial value outside of the local areas. A closer examination at the physical/chemical properties of these products (antibacterial and antioxidant potential, relatively high fiber content and its gelatinization and binding capabilities) indicates a potential broader use of these in both military and commercial foods. Under this program scientists looked at the functionality of taro/poi, its application into existing products, and its development into potentially unique military/commercial food items. This is a progress report on one aspect of the program: the antibacterial potential of fermented taro.

Taro as a food product has many interesting properties to it, one of which is its natural fermentation. Poi, the natural fermentation product of taro, is a traditional staple food in Hawaii. In ancient times poi comprised a large portion of the Hawaiian diet, today it is still sold commercially in Hawaii. The interest in taro is in the antimicrobial aspects of the fermentation product. The study of the antimicrobial aspects of taro can be divided into four phases. They are:

Phase I, to determine the antimicrobial properties of the natural fermentation product, poi, with particular emphasis on the presence of bacteriocins.
Phase II, to determine the suitability of taro as a growth medium for the cultivation of food safe bacteria capable of producing bacteriocins.
Phase III, to determine feasibility of adding the natural poi product as well as other fermented taro products, produced in phase II, to military rations.
Phase IV, to evaluate the efficacy of fermented taro on the growth activities of targeted foodborne spoilage/pathogenic organisms in specific foods.

The interest in poi is to isolate and identify the natural bacteria involved in the fermentation process and to determine if bacteriocins are present in the product. Bacteriocins are natural antimicrobial peptides produced by certain Gram positive bacteria. These bacteriocins in relatively low concentration can inhibit many food spoilage and pathogenic bacteria, thus our interest in bacteriocins as a food preservative. The literature has shown that a number of lactic acid bacteria are present in the sour poi. Based on this, it is believed that taro can be a good medium for various lactic bacteria that produce bacteriocins. In addition to studying the native flora, researchers evaluated other lactic acid bacteria that produce bacteriocins by using the taro as a growth medium. If successful, taro can act as a functional food ingredient for the preservation of various commercial and military foods.

This is the first year progress report of a two-year funded program. The report will cover the progress made during the first year of the study on Phases I and II. This program is supported by congressional funding with emphasis on enhancing the Hawaiian economy by developing products and industries unique to Hawaii.

METHODS

Preparation of Poi

In preparation of taro corms, the corms are first cooked. The corms are steamed or autoclaved for 20 minutes. The outer skin of the corms is then removed using a knife. After the corm is peeled it is cut into approximately 2" x 2" x 2" pieces. The pieces of corm are then put through a meat grinder three times. The fermentation of the prepared poi is usually very rapid. Within the first 24 hours of preparation, the pH changes from 6.3 to a pH of 4.5. Thereafter, the acidity increases gradually until it reaches an average pH of 3.8 by the third day (Moy and Nip, 1983).

Bacteriocin Assay

The bacteriocin activity was determined by a well diffusion assay (Tagg and McGiven, 1971). Before the samples are assayed, the viable bacteria in the sample are heat killed by placing the sample in a water bath at 80° C for 20 minutes. Samples are placed in Trypticase Glucose Yeast Extract (TGE) agar wells and to facilitate diffusion of the bacteriocin, the agar plates were incubated at 2-5° C for 5-6 hrs after application of the bacteriocin sample. Subsequently, TGE agar plates were overlaid with 10 ml of TGE soft agar (0.75%) inoculated with an overnight culture of test organism, *Lactobacillus plantarum* or *Micrococcus luteus,* depending on the bacteriocin being screened. The plates were then refrigerated overnight before incubating at 37° C for 24 hrs. Plates were subsequently examined for zones of inhibition.

RESULTS AND DISCUSSION

Phase I

In Phase I, the objective is to determine the antibacterial properties of the natural fermentation product, poi, with the emphasis on the presence of bacteriocins. The study began by obtaining taro corms from Hawaii that were to be processed to mimic the natural fermentation of taro to poi, as the natives have done for hundreds of years. The corms were ordered from the POI Company (Honolulu, Hawaii) through our contact, Prof. Alvin Huang, University of Hawaii at Manoa. Following the procedure outlined in the methods section, the poi was prepared. Unfortunately, the pH of poi prepared never fell below 5.8 indicating the natural fermentation was being inhibited for some reason. The pH expected was approximately 4.2 (Moy and Nip, 1983). It was determined that we were working with adulterated corms, because they had been treated with chlorine before shipment. This is the usual procedure for any produce being sent out of Hawaii to the continental United States. Due to the treatment, the native flora of the corm was altered thus preventing the natural fermentation of the taro.

This fact has made it more difficult to isolate the native bacteria responsible for the fermentation and has hampered our progress in Phase I. The plan was to isolate the different organisms present during the natural fermentation and determine if any produced bacteriocins. Instead we have obtained freeze-dried samples of the poi from Professor Huang in Hawaii at

different points in the fermentation. Five bacteria have been isolated from these freeze-dried samples with a preliminary identification of the bacteria using the Bio Log System (Hayward, CA). The genus of the bacteria isolated were tentatively identified as two *Leuconostoc* , two *Enterococcus* species and *Streptococcus* species. Because the Bio Logs database has a clinical bias to it, we are calling these identifications preliminary. The Bio Log system provides a reference or fingerprint of the five bacteria. The Bio Log System consists of 95 different wells each containing a carbon source. A color change occurs in the wells in which the carbon source is metabolized giving a fingerprint of the unknown bacteria. Once we establish which of these bacteria are involved in the fermentation of taro further testing will be done on those bacteria for final identification.

There is concern that the bacteria isolated thus far may not represent the bacteria involved in the fermentation of taro to sour poi. The bacteria tentatively identified, with exception of the *Streptococcus* species, are not the usual bacteria found in fermented poi product based on the literature (Allen and Allen, 1933, and Huang et. al., 1994). Due to the handling procedures, the preparation and transport of the freeze-dried samples, the natural flora may be altered. Because of this factor a trip to Hawaii will be made to isolate bacteria from fresh corms and the freshly prepared fermented poi. In this way we will have total control of the sampling procedures, and the preparation and transport of the bacteria isolated. Using the Bio Log System, the bacteria isolates will be identified and compared with the bacteria previously identified. This will allow the verification of the previous results and to identify other bacteria that maybe responsible for the natural fermentation of taro.

Phase II

The adulterated taro corms received from Hawaii were evaluated for use as a medium for bacteriocin produces. The fresh corms were prepared by peeling the corms, cut into small (2" x 2" x 2") pieces that are ground in a meat grinder (three passes). Four concentrations were made 100%, 50%, 25% and 12.5% taro. The taro was diluted with distilled water. The taro is then sterilized in the autoclave. The sterilized taro was inoculated with a bacteriocin producer *Streptococcus sp. ATCC 10035*, a diplococcin producer. Each sample has a total volume of 100 ml. The inoculum was 100 ul of an overnights culture of TGE, approximately 10^4 cells/ml. To determine if growth has occurred in the flasks, the pH was monitored (Table 1). The controls used in the experiment were uninoculated flasks containing taro at each concentration. Flasks were incubated at 37° C for 40 hrs.

Table 1. The pH of four concentrations of taro inoculated with *Streptococcus sp. ATCC 10035*.

Sample	100% Taro	50% Taro	25% Taro	12.5% Taro
Control	6.41	6.27	6.45	5.79
Streptococcus	5.28	4.63	4.22	3.92

From the results it appears that taro will support growth of the bacteriocin producer *Streptococcus sp. ATCC 10035*. The lower the concentration of taro, the lower the pH of the flask. The lower pH indicates better growth of the bacteria. This maybe due to the fact that the

lower percentage of taro has a lower viscosity, enhancing the growth of the bacteria. The lower viscosity of the medium may allow better distribution of the non-motile bacteria providing better access to the available nutrients in taro. The data establishes taro as good medium for lactic acid bacteria without the need for any supplements to the medium.

Table 2. Composition data of taro corms, based on fresh-peeled weight (100g)

Component	Typical Poi Taro	Range (varieties of *C. esculenta*)
Moisture	70 g	62-75 g
Protein	0.7 g	0.5-2.1 g
Lipids	0.2 g	0.1-0.4 g
Starch	24 g	22-28 g
Granule size	3 microns	2.6-4.3 microns
Amylose	4.4 g	1.5-5.3 g
Amylopectin	19.7 g	20.5-22.7 g
Phosphates	0.005 g	NA
Sugars		
Sucrose	1.2 g	0.8-1.8 g
Fructose	0.05 g	0.02-0.4 g
Glucose	0.10 g	0.04-0.12 g
Maltose	0	0-0.08 g
Dietary Fibers		
Soluble	1.3 g	0.3-1.8 g
Insoluble	2.3 g	1.4-3.7 g
Vitamins		
Vitamin A	10 I.U.	0-50 I.U.
Vitamin C	15 mg	5 mg-50 mg
Vitamin B1	0.05 mg	0.03-0.10 mg
Vitamin B2	0.06 mg	0.02-0.10 mg
Niacin	0.6 mg	0.5-1.0 mg
Organic Acids		
Succinic/Malic Acid	160 mg	80-400 mg
Oxalic Acid	100 mg	20-400 mg
Lactic Acid	2 mg	0-10 mg
Citric Acid	60 mg	20-100 mg
pH	6.4	6.2-6.7
Minerals		
Sodium	10 mg	1-16 mg
Potassium	350 mg	300-900 mg
Calcium	35 mg	20-250 mg
Maganesium	40 mg	20-120 mg
Phosphorous	90 mg	40-140 mg
Iron	1.7 mg	0.5-2.3 mg
Zinc	0.2 mg	0.1-4.7 mg

The previous table is a list of components that make up taro (Table 2). Prof. Huang has put together this comprehensive table of components that make up taro. The data collected from various references have been converted based on 100 grams of fresh-peeled taro corms. The first column is data for wetland poi taro corms, and the second column is data for other taro within the *Colocasia esculenta* species.

The adulterated corms seemed to provide a good growth medium even after chlorine treatment. To reduce the cost and waste of corms that can go bad if not used within a few weeks time, the corms were freeze-dried. By peeling the skin off the fresh corms, cutting into 2" x 2" x 2" pieces and grinding in a meat grinder (three passes) the taro was preserved by freeze-drying the material. This freeze-dried taro is kept in a freezer until needed and reconstituted by adding appropriate amount of water. Usually for preparing medium, the freeze-dried taro is autoclaved to cook and sterilize the product. The remainder of the experiments in this report uses the freeze-dried taro just described unless stated otherwise.

Since taro can support the growth of *Streptococcus sp. ATCC 10035,* the next question to be answered is whether there is bacteriocin production and, if so, the activity level. Freeze dried taro concentrations were made of 26%, 13%, 6.5%, 3.25%, 1.63% and 0.81%. The volume in each flask was 100ml and appropriate concentration made based on weight/volume. The lower concentrations of taro were included in the study to see if lower concentrations would support growth and if a bacteriocin was produced. The *Streptococcus* inoculum was 10^4 cells/ml. Table 3 shows the pH of each flask at incubation times of 22, 46 and 70 hrs and bacteriocin production at 70 hrs.

Table 3. The pH of flasks containing various concentrations of taro inoculated with *Streptococcus sp. ATCC 10035* and analyzed for bacteriocin production.

Samples	26% Taro	13% Taro	6.5% Taro	3.25% Taro	1.63%Taro	0.81%Taro
22 hrs	5.24	4.77	4.72	4.04	3.82	3.84
42 hrs	5.17	4.57	4.26	3.77	3.54	3.53
70 hrs	5.28	4.68	4.25	3.83	3.66	3.67
bacteriocin	ND*	ND*	ND*	ND*	ND*	ND*

* ND No detectible bacteriocin

As in a previous experiment (Table 1), the pH of the cultures were lower with lower taro concentrations. Even at the lowest concentration of taro (0.81%) there is good growth based on the pH. However it appears that with the growth of the bacteria no detectible bacteriocin was produced in any of the cultures tested. This is surprising since the production of the bacteriocin occurs during the growth phase of the bacteria and is a constitutive product. The taro is either inhibiting the production of the bacteriocin, the bacteriocin is binding to the taro inactivating it or the growth of the bacteria is not as good as the low pH suggests.

To determine if the *Streptococcus* does grow well in the taro, a viable cell count was made of inoculated taro culture. Samples of the taro culture were taken at times 0, 8, 24, 30 and 48 hrs (Table 4). The taro concentration was 5% and incubated at 37°C. Control was an uninoculated flask of 5% taro.

It appears from the results that 5% taro supports good growth of *Streptococcus sp. ATCC 10035*. The growth curve peaked at 24 hrs with 1.2×10^8 cells/ml. The culture was analyzed for

bacteriocin and no detectible inhibition of the test bacteria, *Lactobacillus plantarum*, was exhibited. The *Streptococcus* used for the inoculum was screened for bacteriocin production to determine if the bacteria still produced the bacteriocin. The test was positive. It can be concluded from these results that the lack of bacteriocin production is not the result of poor growth of the bacteria in taro.

Table 4. Viable cell count and pH of 5% taro inoculated with *Streptococcus sp ATCC 10035*.

Samples	0 Time	8 hrs	24 hrs	30 hrs	48 hrs
Control (pH)	6.4	6.2	6.2	6.2	6.15
Streptococcus (pH)	6.4	6.0	4.9	4.7	4.58
Streptococcus (viable cells)	2.0×10^4	2.0×10^7	1.2×10^8	1.0×10^8	1.0×10^8

A simple experiment was prepared to determine if taro contained a factor which inhibits the production of the bacteriocin from *Streptococcus sp. ATCC 10035*. Trypticase glucose yeast extract medium (TGE) is the medium normally used to grow the bacteria and supports the production of bacteriocin. Three media were made TGE, TGE/5% taro, and 5% taro. Each of these media was inoculated with *Streptococcus* and incubated for 24hrs at 37° C. The media were then analyzed for bactreriocin production. Results showed that TGE and TGE/5% taro supported growth and bacteriocin production while 5% taro had good growth but no detectible bacteriocin. These results indicate that there is no inhibitory factor in taro that prevents the production of the bacteriocin. However, the taro maybe missing an essential ingredient that prevents the production of the bacteriocin.

The TGE is composed of 1 % trypticase, 1 % glucose, 1 % yeast extract, 0.2 % Tween 80, 0.02 mM magnesium and 0.033 mM manganese. An experiment was designed with eight different media to determine if there is a component(s) in TGE that is essential for bacteriocin production. The two control media were TGE and 5% taro. The other six media all contained 5 % taro plus one of the components that makes up TGE. The media were inoculated with *Streptococcus* and incubated at 37° C for 24 hrs (Table 5).

Table 5. The pH and bacteriocin production in media containing 5 % taro plus one component of TGE media, inoculated with *Streptococcus sp. ATCC 10035*.

Sample	pH	Bacteriocin Production
TGE	4.19	+
5 % taro	4.42	ND*
5 % taro + 1 % trypticase	5.23	+
5 % taro + 1 % glucose	4.61	ND*
5 % taro + 1 % yeast extract	4.46	+
5 % taro + 0.2 % Tween 80	4.40	ND*
5 % taro + 0.02 mM Mg	4.47	ND*
5 % taro + 0.033 mM Mn	4.57	ND*

* ND No detectible bacteriocin

The results indicate that the *Streptococcus* bacteriocin was not produced in taro unless trypticase or yeast extract was present in the media. Trypticase is a pancreatic digest of casein that provides to the medium an additional nitrogen source. Yeast extract is the water-soluble portion of autolyzed yeast providing the medium a source of naturally occurring B-complex vitamins. It appears that if a bacteriocin is to be produced by *Streptococcus sp. ATCC 10035* in taro, a supplement will have to be added to the medium.

Another bacterium, *Lactococcus lactis subsp. lactis ATCC 11454,* was tested to determine if it grows and produces a bacteriocin in taro. This bacteria produces nisin, a bacteriocin that has been approved by the FDA for use as a preservative in certain foods. The *L. lactis* was inoculated into a 5 % taro medium. The bacteria grew well in the taro medium incubated at 37° C for 40 hrs, producing a pH of 4.16. Analyzing the culture for bacteriocin, nisin was produced, inhibiting the test bacteria *Micrococcus luteus ATCC 10240.* It appears from this data that *L. lactis* can grow and produce a bacteriocin in taro without supplementing the medium.

In an experiment to determine the minimum concentration of taro needed to produce nisin from *L. lactis,* five different concentrations of taro were prepared. *L. lactis* was inoculated into taro concentrations of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 %. The taro broths were analyzed for nisin production (Table 6). Growth of *L. lactic* was at 37° C for 24 hrs.

Table 6. Determine nisin production by *Lactococcus lactis* in different taro concentrations.

Sample	Bacteriocin Production
0.5 % Taro	**
1.0 % Taro	****
2.0 % Taro	****
3.0 % Taro	****
4.0 % Taro	****
5.0 % Taro	****

* Size of zone of inhibition

In analyzing the samples for nisin production it appears that any concentration of 1% or higher will produce good growth of the bacteria and similar amounts of nisin without supplementing the taro. At a 0.5 % taro there appears to be a limiting nutrient that reduces the production of nisin to half the amount detected for taro concentrations of 1 % or higher.

To test the taro as a food preservative in rations, the quantity of taro containing nisin is to be upscaled before testing in Phase III begins. The goal was to produce 100 g of freeze-dried taro containing nisin. The activity of nisin in the taro was tested against the test organism *M. luteus* using the bacteriocin assay. Three liters of a 5 % taro solution were made with a liter of the taro in each of three flasks. Each flask contained a starting inoculum of approximately 10^4 cells/ml of an overnight culture of *L. lactis*. The flasks were incubated at 37° C for 30 hrs. The 30 hrs taro culture was heat killed at 80° C for 35 minutes, rather then the usual 20 minutes, due to the large volume of liquid. The taro culture was then freeze-dried. Bacteriocin analysis of the taro culture showed no difference in nisin activity before or after freeze-drying, indicating the relative stability of the bacteriocin. The 3 liters of taro freeze dried yielded approximately 100 g

of fermented taro. The activity of the nisin in the freeze-dried taro against *Micrococcus* was approximately 8,000-10,000 activity units (AU)/g. The activity of the nisin in the freeze-dried fermented taro appears to be high enough to begin testing the material in military rations.

CONCLUSIONS

Phase I of the program has progressed more slowly then expected due to the mandatory chlorine treatment of the exported taro corms by local authorities. This difficulty has been circumvented to some degree by sampling freeze dried taro samples from Hawaii. From the freeze-dried taro, bacteria have been isolated that may be responsible for the natural fermentation of poi. The isolates have been used to inoculate sterile taro medium and the medium has exhibited the expected drop in pH characteristic of the natural fermentation of poi. Further work in identifying these isolates is underway and additional isolates are being screened from the freeze-dried samples. A trip to Hawaii has been arranged to isolate bacteria from fresh taro corms to verify the current isolates as well isolating any new fermenting bacteria.

The progress in phase II has been very good. It has been determined that taro can support growth of bacteria that produce bacteriocin and the concentration of taro needed to support the production of bacteriocins can be at very low levels (1% of the freeze dried taro). Results have shown that not all bacteriocin producers can produce bacteriocin without supplementing the taro medium with an additional nitrogen source. *L. lactis*, a nisin producer, produces substantial quantities of the bacteriocin in taro without any supplements to the medium. The fermented taro containing nisin can be freeze-dried, in preparation for its incorporation into rations, without any loss of inhibitory activity. The material is ready for phase III testing in which the taro containing nisin will be incorporated into a military ration to determine its effectiveness as a food preservative.

Future work will concentrate on evaluating the natural bacterial isolates from poi for bacteriocin production. Other bacteriocin producers, with and without supplements to the taro, will be evaluated for production. The most promising taro bacteriocin producers will be incorporated into military rations to determine their effectiveness as food preservatives against various food spoilage bacteria and pathogens.

This document reports research undertaken at the U.S. Army Soldier and Biological Chemical Command, Soldier Systems Center, Natick, MA, and has been assigned No. NATICK/TR-02/018 in a series of reports approved for publication.

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